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D-Xylose (D-glucose) isomerase from *Arthrobacter* strain N.R.R.L. B3728

Purification and properties

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D-Xylose (D-glucose) isomerase was purified to homogeneity in yields of approx. 1 g/kg of wet cells from a strain of *Arthrobacter* that produces it as about 10% of total soluble protein. It is a tetramer of identical 43114 Da subunits containing a preponderance of acidic residues and no cysteine. Partial protein sequences were determined as a step to gene cloning. It requires Mg^{2+}, Co^{2+} or Mn^{2+} for activity, Mg^{2+} being best; Ca^{2+} is an inhibitor, competitive with Mg^{2+}. It is a good D-glucose isomerase with $k_{cat.}$ 1200 min^{-1} at pH 8 at 60 °C, which is higher than that of any other enzyme of this class. L-Arabinose, D-ribose and D-lyxose are poor substrates, with $k_{cat.}$ 78, 31 and 3.7 min^{-1} respectively at pH 8 at 30 °C, compared with 533 min^{-1} for D-xylose. Xylitol is a true competitive inhibitor for D-xylose (K_i 0.3 mM), but D-sorbitol shows mixed inhibition (K_i 6.5 mM). For D-fructose the pH optimum at 60 °C is 8, and at pH 7 the Arrhenius activation energy is 75 kJ/mol over the range 30–70 °C.

INTRODUCTION

D-Xylose (D-glucose) isomerase (EC 5.3.1.5) (Reynolds, 1973) was developed by Imperial Chemical Industries Ltd. (I.C.I.) in the late 1970s as an immobilized glucose isomerase for production of sweeter high-fructose syrups from glucose syrups derived from starch by α-amylase and glucoamylase. Its advantages are a relatively high side-specificity for glucose, a thermostability sufficient to allow long periods of column operation at 60 °C and a food-compatible requirement for Mg^{2+} only as cofactor, rather than Mg^{2+} plus Co^{2+}, which are needed for enzymes in current commercial use (Barker, 1975; Coker & Venkatasubramanian, 1985). In collaboration with I.C.I., we purified and determined many of the properties of the enzyme from *Arthrobacter* strain N.R.R.L. B3728 (a xylose isomerase-constitutive mutant of the xylose-inducible wild-type strain N.R.R.L. B3724), which produces this enzyme as more than 10% of its total soluble protein (Smith, 1980).

But the advent of protein engineering changes the prospects for novel glucose isomerases, since desirable improvements might be achieved by site-directed mutagenesis based on a detailed tertiary structure. Because of the high cost of the enzyme, current processes use columns of stable immobilized enzymes at 60 °C and pH 7–8, their pH optima, but costs of immobilization and column operation add significantly to the product price. Since the fructose/glucose equilibrium increases with temperature (Takasaki, 1967), a higher-temperature isomerization would yield a sweeter syrup. However, it would then be necessary to operate at lower pH, since undesirable 'browning reactions' occur when glucose is heated at alkaline pH. Hence enzymes with pH optima below 6 and stability above 80 °C are useful protein engineering targets.

The superior properties of the *Arthrobacter* B3728 enzyme made it a good starting point for such a programme. But relatively little was known about xylose (glucose) isomerases, despite their commercial importance for the production of high-fructose syrups and their potential for constructing yeasts able to ferment waste xylose from paper pulp production. Hence basic insights into mechanism, thermostability and the role of electrostatic effects in proteins might also arise from the cloning, structure determination and protein engineering that were necessary to reach the applied targets.

The present paper describes the purification and properties of the wild-type enzyme that form the background to the crystallization (Akins *et al.*, 1986) and tertiary-structure determination (Henrick *et al.*, 1989) and to hypotheses about the catalytic mechanism (Collyer *et al.*, 1990). Another strategic step was the construction of a host vector system for *Arthrobacter* N.R.R.L. B3728 (Shaw & Hartley, 1988). Studies of cloning, sequencing and expression are reported in the following paper (Loving-Anderton *et al.*, 1991).

MATERIALS AND METHODS

Materials

DEAE-Sephacel, DEAE-Sepharose and Sephadex G-200 were obtained from Pharmacia (Hounslow, Middx., U.K.), Ultrogel AcA 34 and Ultrogel AcA 22 were purchased from LKB (Croydon, Surrey, U.K.), and Bio-Gel P-4, Bio-Gel P-6 and Bio-Rad protein reagent were from Bio-Rad Laboratories (Watford, Herts., U.K.). Pentitols, D-fructose (low in D-glucose), D-xylose and D-sorbitol were from Cambrian Chemicals (Croydon, Surrey, U.K.). Iodo[^{14}C]acetic acid was purchased from Amersham International (Amersham, Bucks., U.K.). Urea (enzyme grade, ultra-pure) was obtained from BRL (Bethesda, MD, U.S.A.). All other reagents were purchased from BDH Chemicals (Poole, Dorset, U.K.), Fisons (Loughborough, Leics., U.K.) or Sigma Chemical Co. (Poole, Dorset, U.K.).

Arthrobacter strain N.R.R.L. B3728 (Reynolds, 1973) was from the American Type Culture Collection. Media and culture conditions were as in Shaw & Hartley (1988).

Enzyme assays and steady-state kinetics

In all cases conditions were chosen that yielded initial rates (v) that were unequivocally first-order versus time. Controls lacking

Abbreviations used: ABTS, diammonium 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate); PMSF, phenylmethanesulphonyl fluoride.

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enzyme were made to correct for non-enzymic isomerizations. Michaelis constants (K_m), inhibition constants (K_i) and maximum velocities ($V_{max.}$) were determined by linear regression from plots of [S]/v versus [S] (Hofstee, 1952), with at least eight points for each set of assays.

D-Xylose isomerase assay. The standard assay used *Klebsiella aerogenes* D-arabitol dehydrogenase to couple the D-xylulose produced to the oxidation of NADH, monitored at 340 nm in a Cary recording spectrophotometer. Assays were in cuvettes of 1 cm light-path at 30 °C containing the isomerase sample (5–20 μg) plus 1 unit of dehydrogenase in 1 ml of 100 mM-D-xylose / 0.33 mM - NADH / 30 mM - $MgCl_2$ / 100 mM - Tris / HCl buffer, pH 8.0. D-Arabitol dehydrogenase is present in excess in this assay. One unit of D-xylose isomerase catalyses the formation of 1 μmol of NADH/min in this assay system.

The *K. aerogenes* D-arabitol dehydrogenase was purified from an *Escherichia coli* strain that harbours a bacteriophage causing superproduction of this enzyme. One unit causes formation of 1 μmol of NADH/min in an assay system comprising 50 mM-D-arabitol/0.83 mM-NAD^+/100 mM-potassium phosphate buffer, pH 7.0, at 30 °C (Neuberger *et al.*, 1979).

D-Ribose isomerase assay. An analogous assay was used in which an excess of D-ribitol dehydrogenase (Rigby *et al.*, 1974) utilizes NADH to convert the D-ribulose formed into D-ribitol.

D-Lyxose isomerase assay and D-arabinose isomerase assay. Since the respective ketoses corresponding to D-lyxose and D-arabinose are D-xylulose and D-ribulose, the above assays were used to determine isomerase activity against these two substrates.

D-Fructose isomerase assay (Werner *et al.*, 1970). This was the most reliable assay for hexose isomerase activity. The standard assay used enzyme added to 2 ml of 1.25 M-fructose in 30 mM-$MgCl_2$/50 mM-Tris/HCl buffer, pH 8.0, at 30 °C or 30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0, at 60 °C. At intervals, duplicate 100 μl samples were added to 100 μl of 15% (w/v) trichloroacetic acid in 10 ml plastic tubes to stop the reaction. A 5 ml portion of chromogen [diammonium 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS)] containing glucose oxidase and peroxidase (BCL kit, Test-combination glucose; GOD–PERID method) was added to each tube, mixed and left to stand at room temperature for 30 min, and the absorbance was measured at 420 nm. The glucose formed is converted by glucose oxidase into gluconate and H_2O_2, and peroxidase converts the latter into a coloured complex with the chromogen ABTS. Standard curves were constructed for glucose from 0 to 500 nmol of glucose. The colour values did not vary from batch to batch of reagent. One unit of fructose isomerase forms 1 μmol of glucose/min under these assay conditions.

Cysteine/carbazole assays. These were used in discontinuous assays to measure ketose formation from aldoses (Dische & Borenfreund, 1951). Samples containing suitable concentrations of aldose and pure enzyme (for D-glucose 330 μg/ml at 30 °C or 31 μg/ml at 60 °C; for L-arabinose 430 μg/ml at 30 °C) were incubated in 30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0. At 10–15 min intervals, triplicate 700 μl samples were withdrawn and added to 300 μl portions of 15% (w/v) trichloroacetic acid to stop the reaction. To 0.5 ml of each sample, 0.1 ml of freshly prepared cysteine hydrochloride (1.5%, w/v), 3 ml of conc. H_2SO_4 (72%, v/v) and 0.1 ml of carbazole in ethanol (0.12%, w/v) were added. After thorough mixing and incubation at room temperature for 60 min, the absorbance at 540 nm was measured against a reagent blank. The ketose concentrations were measured by using relevant aldose and ketose calibration curves; the D-glucose/D-fructose absorbance ratio was 1:80. For L-arabinose isomerization assays, D-xylose/D-xylulose calibration curves were used, since no L-ribulose was available.

Enzyme purification

A 300-litre aerobic culture of *Arthrobacter* strain N.R.R.L. B3728 was grown at 30 °C to late exponential phase by Imperial Biotechnology Ltd. in a 600-litre fermenter on a medium containing $(NH_4)_2HPO_4$ (6 g/l), KH_2PO_4 (2 g/l), D-glucose monohydrate (57.8 g/l), $MgSO_4,7H_2O$ (1.2 g/l), corn-steep liquor (12 g/l) and silicone RD anti-foam (0.1 g/l) controlled at pH 7 by adding aq. NH_3. Cell yields were about 25 g dry wt./l. The cells were spun down in a Sharples model AS-6 continuous-flow centrifuge, and the cell paste was stored at −20 °C.

Routinely, a 300 g portion of the frozen cell paste was thawed into 300 ml of 10 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride (PMSF)/50 mM-Tris/HCl buffer, pH 7.5, and the cells were pelleted by centrifugation at 27000 g for 30 min. The resulting washed cell paste was resuspended in 900 ml of buffer, 300 mg of lysozyme-Cl was added and the suspension was incubated at 37 °C with shaking for 2–3 h. Then DNAase 1 (10 mg) and $MgCl_2$ (to 30 mM final concentration) were added with occasional mixing and incubated for a further 1 h at 37 °C. All further steps were performed at 4 °C.

The cell debris was removed by two sequential centrifugations for 60 min each at 27000 g, yielding 700 ml of supernatant. After dialysis against 10 mM-$MgCl_2$/150 mM-NaCl/0.1 mM-PMSF/50 mM-Tris/HCl buffer, pH 7.5, the supernatant was applied to a DEAE-Sephacel column (500 ml) that had been equilibrated in the same buffer. The enzyme was eluted by a gradient of NaCl to a final concentration of 300 mM in this buffer.

The fractions containing enzyme activity were concentrated to 50 ml by Amicon PM30 membrane ultrafiltration and further purified by gel filtration on a column of Ultrogel AcA 34 that had been equilibrated in 10 mM-$MgCl_2$/0.1 mM-PMSF/100 mM-Tris/HCl buffer, pH 7.5. Enzyme fractions judged to be pure by constant specific activity and SDS/PAGE were combined and concentrated by ultrafiltration to about 30 mg of protein/ml. The protein at this stage was essentially pure; gel-filtration h.p.l.c. gave no significant further purification.

The concentrated protein solution was filtered (0.2 μm-pore-size membrane) and stored in 250 μl portions at 4 °C in evacuated sealed tubes. Enzyme for protein chemical studies was dialysed against 100 mM-NH_4HCO_3/0.1 mM-PMSF (five changes of 100 vol. each) followed by Milli-Q water (two changes), freeze-dried and stored at −20 °C.

Protein chemistry methods

Determination of protein concentrations. Protein concentrations were determined by using the Bio-Rad assay kit or a modified Lowry assay (Miller, 1959), with BSA as standard. Protein contents in whole cells and cell-free extracts were measured by the biuret assay (Herbert *et al.*, 1971). For purified enzyme, $A^{0.1\%}_{1cm}$ 0.92 at 280 nm was used.

SDS/PAGE. PAGE in the presence of SDS (Laemmli, 1970) was carried out at room temperature in 12.5% (w/v) polyacrylamide slab gels (15 cm × 15 cm × 0.15 cm). Samples were dried *in vacuo*, dissolved in 30 μl of SDS sample buffer [1% (w/v) SDS/50% (v/v) glycerol/0.1% (w/v) Bromophenol Blue/15 mM-Tris/HCl buffer, pH 6.5] and heated at 105 °C for 2 min before loading. R_F values were determined relative to the Bromophenol Blue dye front. Gels were stored in 10% (v/v) acetic acid.

For PAGE under native conditions the resolving gel contained 7% (w/v) acrylamide. All other solutions, electrophoresis buffer and sample buffer were the same as for SDS/polyacrylamide gels, except that SDS was omitted throughout.

Gels were soaked in 0.3% (w/v) Coomassie Brilliant Blue

R250 in acetic acid/methanol/water (2:9:9, by vol.) for 6–8 h at 22 °C to stain for protein, and destained over several days at 22 °C in acetic acid/methanol/water (2:9:9, by vol.).

Xylose isomerase activity in gels was detected by the method of Yamanaka (1975). Gels were soaked in 250 ml of 0.1 M-D-xylose/30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0, at 37 °C for 30 min, washed once with distilled water to remove excess substrate and incubated in the dark in 200 ml of 1 M-NaOH containing triphenyltetrazolium chloride (1 mg/ml). Red bands formed in the region of the active material; the reaction was terminated by soaking the gel in 2 M-HCl.

Determination of molecular mass by gel filtration. (*a*) A Sephadex G-150 column (3.2 cm internal diam. × 80 cm) was loaded with D-xylose isomerase, pyruvate kinase, fumarase (pig heart), lactate dehydrogenase (rabbit muscle), *K. aerogenes* ribitol dehydrogenase, *E. coli* alkaline phosphatase, BSA, ovalbumin and Blue Dextran and eluted with 10 mM-$MgCl_2$/0.1 mM-PMSF/100 mM-Tris/HCl buffer, pH 7.5. Fractions (4 ml) were collected. The void volume was determined with Blue Dextran. The elution positions of the enzymes were identified by assaying enzyme activity; BSA and ovalbumin were located by monitoring the absorbance at 280 nm and SDS/PAGE of peak fractions.

(*b*) A Du Pont Zorbax GF-250 column (9.4 mm internal diam. × 25 cm) was loaded with D-xylose isomerase, apo-ferritin, α-amylase, yeast alcohol dehydrogenase, phosphorylase *b*, BSA, ovalbumin, Blue Dextran and L-tyrosine and eluted with 5 mM-magnesium acetate / 2.5 mM-EDTA / 0.1 mM-PMSF / 100 mM-potassium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected. The elution positions of Blue Dextran, proteins and L-tyrosine were located by monitoring the absorbance at 280 nm.

Determination of isoelectric point. The isoelectric point of the enzyme was determined by electrofocusing PAGE, with the LKB Ampholine system and more recently with the Pharmacia PHAST IEF pH 3–9 system.

Amino acid analysis. Triplicate desalted xylose isomerase samples were hydrolysed at 105 °C in evacuated sealed tubes with 50 μl of 6 M-HCl containing phenol and 2 nmol of norleucine for periods of 24, 48, 72 and 96 h. Samples were analysed on a Durrum D500 amino acid analyser by Mr. K. Edwards (M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.) or on a Beckman 121MB amino acid analyser by Mr. D. Featherbe (Department of Biochemistry, Imperial College of Science and Technology, London, U.K.). Values for serine and threonine were extrapolated to zero hydrolysis time, and those for valine and isoleucine were extrapolated to infinite hydrolysis time. Values for methionine were estimated as the sum of methionine and methionine sulphone and extrapolated to zero hydrolysis time. Tryptophan and tyrosine were determined spectrophotometrically by the method of Beaven & Holliday (1952). For the other amino acids, the values obtained for all four hydrolysis times were averaged. Triplicate samples of protein oxidized with performic acid (Hirs, 1956) were analysed on a Beckman 121MB amino acid analyser to determine the cysteine/half-cystine content of the enzyme.

Carboxylation of methionine residues. This was carried out with iodo[^{14}C]acetic acid in 8 M-urea (Stark & Stein, 1964). Any methionine sulphoxide formed during purification and storage of the protein was reduced by incubating a 3 mg/ml solution in 5% (v/v) 2-mercaptoethanol/6 M-guanidinium chloride/200 mM-Tris/HCl buffer, pH 8.2, for 22 h at 22 °C. After dialysis against aq. 5% (v/v) formic acid, the reduced protein was freeze-dried.

The fully reduced D-xylose isomerase (175 mg) was dissolved in 70 ml of 8 M-urea and allowed to react with 5.33 mmol of iodo[^{14}C]acetic acid (0.13 μCi/μmol) in 20 ml of 8 M-urea at 22 °C for 96 h. The protein solution was dialysed against several litres of 100 mM-NH_4HCO_3 at 22 °C. The degree of incorporation of ^{14}C from iodo[^{14}C]acetate into protein was greater than 95% of the expected value.

Sequencing of tryptic-digest peptides. The denatured ^{14}C-labelled protein (2 mg/ml in 100 mM-NH_4HCO_3) was digested for 5 h at 37 °C with trypsin (0.04 mg/ml) purified on an affinity column of chick ovomucoid coupled to CNBr-activated Sepharose. The tryptic digest was freeze-dried overnight and then applied to a Bio-Gel P-4 (100–200 mesh) column (2.4 cm × 82 cm) equilibrated and eluted with 50 mM-NH_4HCO_3 at 22 °C. The absorbance at 230 nm and ^{14}C radioactivity of the fractions were measured and suitable fractions were combined and freeze-dried. Eight pools were thus obtained. Peptides from each pool were further purified by using a combination of ion-exchange chromatography and/or reverse-phase h.p.l.c. The purity of peptides was established by amino acid analysis and *N*-terminal determination by using the dansyl technique of Gray (1972). Peptides judged to be pure were sequenced by the manual dansyl–Edman procedure of Gray (1967).

Determination of the *N*-terminal sequence. The *N*-terminal sequence of D-xylose isomerase was determined manually by the dansyl–Edman method of Gray (1972). Usually, ten cycles were possible by this method. The first 20 residues from the *N*-terminus were determined by the automated gas-phase Edman method with the CI 4000 gas-phase sequencer by Mr. I. Blench (Department of Biochemistry, Imperial College of Science and Technology, London, U.K.).

RESULTS AND DISCUSSION

Purification of D-xylose isomerase from *Arthrobacter* B3728

Table 1 summarizes the results of a typical purification from 250 g of frozen cell paste as described above. The original purification protocol of Smith (1980) was essentially similar but used first DEAE-Sepharose ion-exchange chromatography, then Sephadex G-200 gel filtration.

Table 1 suggests that the enzyme represents about 5% of the total cell protein and 10% of the total soluble protein; some selective purification is achieved by the lysozyme extraction procedure. For comparison, yields of enzyme from fully xylose-induced cultures of strain B3724 correspond to about 5% of total soluble protein. This implies that the operon has powerful promoter and translation sequences, but also that the B3728 strain is a constitutive superproducer.

The modified purification protocol described above yields about 1 g of very pure protein from 1 kg of cell paste. The enzyme eluted from Sephadex G-200 or Ultrogel AcA 34 was homogeneous as judged from SDS/PAGE and native PAGE. The specific activity was 2.3 units/mg with D-fructose at 30 °C and 12.5 units/mg with D-xylose at 30 °C.

Molecular mass and subunit structure

The original studies by Smith (1980) gave a subunit molecular mass of 47000 Da from calibrated SDS/PAGE gels and a native molecular mass of 185000 ± 5000 Da from gel filtration on Sephadex G-150 (mean ± range, $n = 2$). Salomon & Ellenrieder (1987) obtained a native molecular mass of 151000 Da for the D-xylose isomerase purified from the related *Arthrobacter* strain B3727. However, the coding sequence of the structural gene predicts a subunit size of 43114 Da and this fits the tetrameric tertiary structure very satisfactorily (Henrick *et al.*, 1989).

These discrepancies prompted a thorough re-investigation of the apparent molecular mass. Sephadex G-150 gel filtration with seven marker proteins and Blue Dextran gave a native molecular

Table 1. Purification and yield of D-xylose isomerase from *Arthrobacter* strain B3728

For experimental details see the text. The results are from 250 g of cell paste. Fractions were assayed against 0.1 M-D-xylose/30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0, at 30 °C.

Purification step	Total activity (units)	Total protein (g)	Specific activity (units/mg)	Yield Step (%)	Yield Total (%)	Purification (fold) Step (%)	Purification (fold) Total (%)
Lysis supernatant	5775	9.24	0.63	100	100	1.0	1.0
DEAE-Sephacel pool (concentrated)	3465	0.33	10.0	57	57	15.9	15.9
AcA 34 pool (concentrated)	2875	0.23	12.5	83	50	1.25	19.8

mass of 185000 ± 5000 Da, and gel-filtration h.p.l.c. on Du Pont GF 250 columns gave 180000 ± 5000 Da (results not shown). However, in carefully calibrated SDS/PAGE gels (results not shown) the apparent subunit size remained 47000 ± 2000 Da (mean ± S.E.M., $n = 6$). The apparently high subunit molecular mass on SDS/PAGE gels may be due to lower-than-average SDS binding resulting from the high content of acidic residues.

Amino acid composition and isoelectric point

Table 2 shows the amino acid composition derived from triplicate samples, acid-hydrolysed for 24, 48, 72 and 96 h to allow extrapolation to zero time for histidine, serine and threonine and to infinite time for isoleucine, leucine and valine (Smith, 1980). There are large discrepancies in the values of glutamic acid/glutamine (45.2 versus 36), alanine (54.6 versus 43) and serine (18.8 versus 15). The apparently high values can be explained in retrospect by the presence of these amino acids at significant concentrations in the distilled water used in the final purification and freeze-drying experiments. High lysine values (21.5 versus 18) could be due to inadequate resolution between lysine and ammonia peaks during amino acid analysis. Low values of tyrosine (7.1 versus 9) could be due to oxidation by halide ions during hydrolysis in spite of phenol being added to protect tyrosine. Values for valine (14 versus 18) and phenylalanine (20 versus 26) are lower. There are three Val-Phe, one Phe-Val and one Phe-Phe sequences in *Arthrobacter* D-xylose isomerase (Loviny-Anderton *et al.*, 1991). It is possible that the 96 h hydrolysis time was still not sufficient to hydrolyse these bonds completely and hence the values are lower. Tryptophan values were estimated from the spectral ratio in 10 mM-NaOH at 280 nm and 294.4 nm, and the absence of cysteine residues was confirmed by using hydrolysates of performic acid-oxidized enzyme. The low value for ammonia in these analyses indicates that there is a predominance of acidic residues over total basic residues. This is also reflected in the low isoelectric point, pI 3.5, obtained by electrofocusing PAGE in the LKB Ampholine system, and is confirmed by the composition derived from the coding sequence quoted in Henrick *et al.* (1989), which is also shown in Table 2.

N-Terminal and tryptic-digest peptide sequences

The *N*-terminal sequence derived by the manual dansyl–Edman technique was:

Ser-Val-Glx-Pro-Thr-Pro-Ala-

This was confirmed and extended by automated gas-phase sequencing, giving:

Ser-Val-Gln-Pro-Thr-Pro-Ala-Asp-His-Phe-Thr-Phe-Gly-Leu-Trp-Thr-Val-Gly-Trp-Thr-

Table 2. Amino acid composition of *Arthrobacter* B3728 D-xylose isomerase

Values for serine and threonine are extrapolated to zero time and values for isoleucine and valine to infinite time of acid hydrolysis. Values for tryptophan and those in parentheses for tyrosine and tryptophan were derived spectrophotometrically. Absence of cysteine was confirmed by analyses on performic acid-oxidized protein. The subunit molecular mass (43114 Da) and coding sequence composition are from our own data (Loviny-Anderton *et al.*, 1991) as briefly reported in Henrick *et al.* (1989).

Residue	Residues per subunit: Observed	Residues per subunit: DNA sequence
Asp } Asn	45.0	31 } 18
Glu } Gln	45.2	27 } 9
Ser	18.8	15
Thr	20.7	23
Gly	37.8	41
Ala	54.6	43
Val	13.9	18
Leu	38.9	39
Ile	15.2	15
Pro	15.9	17
Met	7.8	9
Cys	0.0	0
Phe	20.4	26
Tyr	6.2 (7.1)	9
His	12.3	13
Lys	21.5	18
Arg	16.3	18
Trp	3.1 (3.6)	5
Amide NH_3	(15–25)	–
Total	394	394

The complete or partial sequences of the following tryptic-digest peptides were determined by the manual dansyl–Edman technique:

(1) Asp-Ala-Glu-Ala-Ala-Ala-Glu-Arg

(2) Asp-Ala-Thr-Glu-Ala-Glu-Arg

(3) Ile-Glx-Glx-Leu-Glx-His-Gly-

(4) Asx-Ile-Val-Gly-Leu-Asx-Pro-

(5) Asx-Leu-Leu-Glx-Asx-Gly-Phe-Pro-

(6) Ala-Asx-Pro-Glx-Val-Glx-Glx-Ala-Met-

(7) Thr-Ser-Gly-Val-Phe-Glx-

(8) Asx-Leu-Met-Asx-Asx-Ser-Ala-

(9) Glx-Leu-Gly-Ala-Tyr-

(10) Gly-His-Gly-

Some of these sequences were used for design of oligonucleotide probes as part of the gene-cloning strategy. Acidic groups were allocated from the mobility of the peptides or dansyl-peptides in high-voltage paper electrophoresis.

Steady-state kinetics and substrate-specificity

Table 3 shows steady-state kinetic parameters for the pure enzyme against various substrates, determined in 30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0. No activity was found against D-arabinose, L-xylose or L-rhamnose. Values of $K_{m(app.)}$ will reflect differences in equilibrium between pyranose, furanose and open-chain forms of the various sugars, but ratios of $k_{cat.}$ presumably reflect only the isomerization step.

Fig. 1 shows the open-chain structures of several sugars together with $k_{cat.}$ values for their isomerization at 30 °C by the *Arthrobacter* B3728 enzyme. As expected, C-2 stereospecificity is uppermost, but C-3 stereospecificity is not absolute (D-ribose), nor is C-4 stereospecificity (L-arabinose).

The small activity towards D-lyxose is puzzling, and we suspected that this might be due to some small D-xylose contamination in the commercial substrate. However, calculations showed that to account for the results this could not exceed 0.04%, even allowing that D-lyxose is a probable competitive inhibitor (Yamanaka, 1969). Since at least 4% of the D-lyxose sample was converted into D-xylulose in an experiment conducted over 36 h, we consider the $k_{cat.}$ to be significant.

The relatively high rates for D-glucose and D-fructose reflect the fact that this is one of the D-xylose isomerases that has been selected as a 'glucose isomerase' to minimize steric constraints at the C-6 position at the active site. In view of the detailed tertiary structures that are emerging for 'glucose isomerases' from *Arthrobacter* (Henrick *et al.*, 1989; Collyer *et al.*, 1990), *Streptomyces rubiginosus* (Carrell *et al.*, 1989), *Streptomyces olivochromogenes* (Farber *et al.*, 1989) and *Actinoplanes missouriensis* (Rey *et al.*, 1988), fine comparisons of their relative kinetics and specificity will be revealing.

Table 3. Kinetic parameters for purified *Arthrobacter* B3728 D-xylose isomerase

Assays were in 30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0.

Substrate	K_m (mM)	$V_{max.}$ (μmol/min per mg)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ ($min^{-1} \cdot mM^{-1}$)
D-Xylose (30 °C)	3.3	12.25	533	162
L-Arabinose (30 °C)	280	1.8	78.3	0.28
D-Ribose (30 °C)	190	0.71	30.9	0.16
D-Lyxose (30 °C)	86	0.086	3.7	0.04
D-Glucose (30 °C)	225	3.2	139	0.619
D-Fructose (30 °C)	170	2.3	100	0.588
D-Glucose (60 °C)	210	27.4	1192	5.68
D-Fructose (60 °C)	250	28.2	1227	4.91

Table 4 compares selected data for some enzymes of this class. Strict comparisons are not possible because of the variety of conditions in which these activities are measured, particularly the nature and concentration of the bivalent metal ions used. However, ratios of $V_{max.}$ towards D-glucose or D-fructose versus D-xylose are illuminating because these must reflect subtle differences in enzyme conformation. In this respect, the *Arthrobacter* enzyme emerges as the best glucose isomerase, particularly since it requires only Mg^{2+} ions and contains no thiol groups, which are prone to oxidation in industrial use.

Competitive inhibition by polyols

Xylitol and D-sorbitol were expected to act as competitive inhibitors, as suggested by their structures (Fig. 1). At 30 °C versus D-xylose under the conditions used in Table 3, the Lineweaver–Burk plots (Fig. 2) show that xylitol inhibition is truly competitive with a K_i of 0.3 mM, whereas D-sorbitol shows mixed inhibition with a competitive K_i of 6.5 mM but also causing a decrease in $k_{cat.}$. This unexpected result suggests that

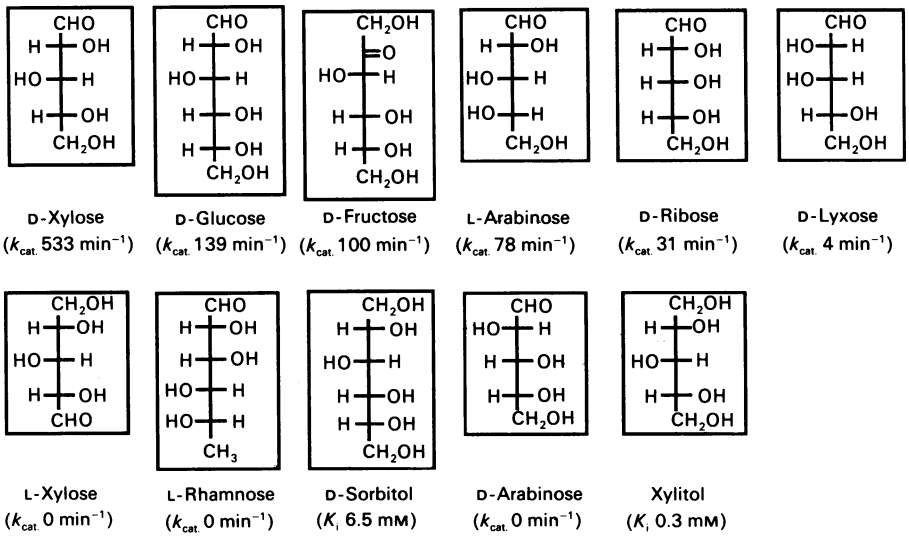

Fig. 1. Open-chain structures and relative specificity of substrates and inhibitors of *Arthrobacter* B3728 D-xylose isomerase

For the substrates (upper row) $k_{cat.}$ values at pH 8 at 30 °C are taken from Table 3. The K_i values for xylitol and D-sorbitol are from Fig. 2.

Table 4. Kinetic parameters for some purified D-xylose (D-glucose) isomerases

Key to references: [a]the present work; [b]Suekane *et al.* (1978); [c]Kasumi *et al.* (1982); [d]Gong *et al.* (1980); [e]Danno (1970).

Bacterial strain	Temperature (°C)	K_m (mM)				$V_{max.}$ (μmol/min per mg)			
		Glucose	Fructose	Xylose	Ribose	Glucose	Fructose	Xylose	Ribose
Arthrobacter B3728[a]	30	225	170	3.3	190	3.2	2.3	12.3	0.71
	60	210	250	–	–	27.4	28.2	–	–
Streptomyces olivochromogenes[b]	60	250	200	33	–	5.3	4.8	21.8	–
Bacillus stearothermophilus[b]	60	220	200	100	–	6.0	5.7	44.5	–
Streptomyces griseofuleus S-14[c]	60	220	–	54	–	17.6	–	44.2	–
Actinoplanes missouriensis[d]	60	1330	1670	–	–	–	–	–	–
Bacillus coagulans[e]	40	90	–	1.1	83	2.9	–	7.3	1.67

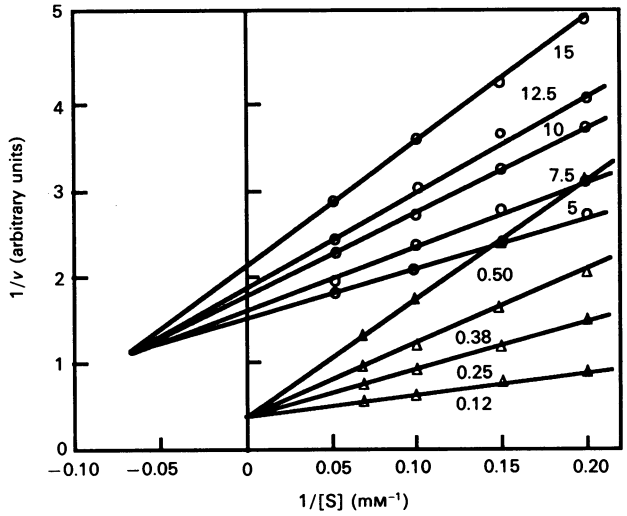

Fig. 2. Inhibition of D-xylose isomerase activity by xylitol and D-sorbitol

The Lineweaver–Burk plots shown are for initial rates (v) at various D-xylose concentrations [S], determined at 30 °C in 30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer, pH 8.0, containing various concentrations (mM) of xylitol (△, lower series) or D-sorbitol (○, upper series).

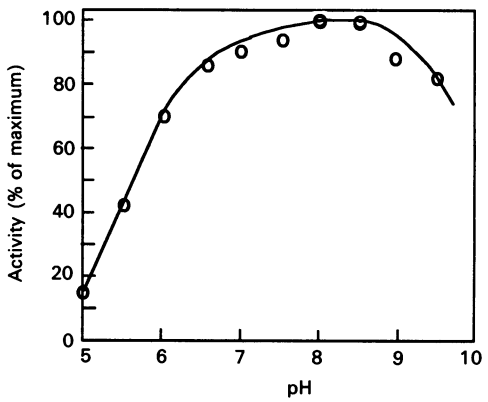

Fig. 3. pH-dependence of D-fructose isomerase activity

Initial rates are shown for 1 M-fructose/30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer adjusted to various pH values at 60 °C. No inactivation of the isomerase was observed during the incubations.

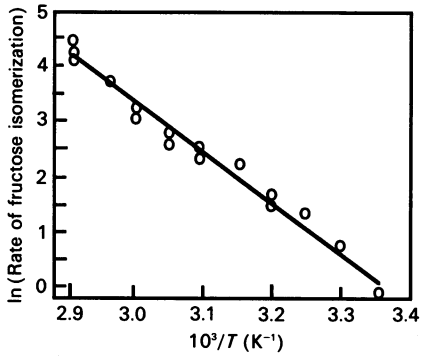

Fig. 4. Arrhenius plot of D-fructose isomerase activity at pH 7.0

Initial rates are shown, with 1 M-D-fructose/10 mM-$MgCl_2$/10 mM-NaCl/100 mM-potassium phosphate buffer and suitable amounts of enzyme, adjusted to pH 7.0 at 25 °C and incubated at various temperatures from 25 °C to 70 °C.

binding of xylitol to one subunit of the tetramer has no effect on the activity of the three others, whereas the steric constraint imposed to accommodate the additional $-CH_2OH$ group in D-sorbitol has adverse effects on the catalytic activity of adjacent active sites. It should be noted that Danno (1970) showed that, for *Bacillus coagulans* enzyme, xylitol, D-mannitol and D-sorbitol showed mixed inhibition against D-xylose, D-glucose and D-ribose, suggesting that in this case each polyol exercises effects across the subunit interfaces.

Effects of pH and temperature on D-fructose isomerase activity

The pH–activity curve (Fig. 3) was determined against 1 M-D-fructose/30 mM-$MgCl_2$/100 mM-Tricine/NaOH buffer over the pH range 5.0–9.5 at 60 °C since that is the temperature of industrial processes. The pH optimum of 8.0 was as reported for the isomerization of D-glucose by the less purified enzyme (Reynolds, 1973) and is similar to that observed for other enzymes of this class. The decrease in activity at acid pH suggests an active-site ionization with pK_a 5.6.

Fig. 4 shows an Arrhenius plot for the activity against 1 M-D-fructose / 10 mM - $MgCl_2$ / 10 mM - NaCl / 100 mM - potassium phosphate buffer, pH 7.0, over the temperature range 25–70 °C. Phosphate buffers were used because they show little variation with temperature. Results above 70 °C are excluded because non-enzymic isomerization rates become significant. The gradient corresponds to an activation energy of 75 kJ/mol. Danno (1970) reported an activation energy of 61 kJ/mol for the isomerization of three aldoses (D-xylose, D-ribose and D-glucose) by the analogous enzyme from *B. coagulans*, but this comparison lacks significance because it concerns the reverse reaction.

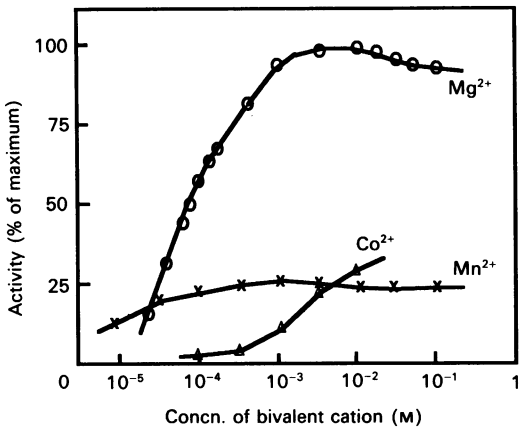

Fig. 5. Dependence of D-xylose isomerase activity on bivalent cation concentration

D-Xylose isomerase activity was determined at 30 °C in 100 mM-Tricine/NaOH buffer. In the case of Mg^{2+} and Mn^{2+} the pH was 8.0; for Co^{2+} pH 7.0 was used and the rate was expressed relative to the maximum rate obtained with Mg^{2+} at the same pH value.

Table 5. Competition by bivalent metal ions for *Arthrobacter* B3728 D-xylose isomerase

The effects of Mg^{2+}, Mn^{2+} and Ca^{2+} on initial rates with 10 mM-D-xylose/100 mM-Tricine/NaOH buffer, pH 8.0, at 30 °C are shown as percentages of the activity observed with 10 mM-Mg^{2+} alone.

Concn. of metal ion (mM)			Activity
Mg^{2+}	Mn^{2+}	Ca^{2+}	(%)
1	–	–	91
10	–	–	100
50	–	–	91
–	10	–	23
1	10	–	25
10	10	–	51
50	10	–	72
–	10	10	16
1	–	1	28
10	–	1	86
50	–	1	91
1	–	10	5
10	–	10	22
50	–	10	53

Requirement for bivalent metal ions

All known D-xylose isomerases require the bivalent cations Mg^{2+}, Co^{2+} or Mn^{2+} for activity, and these cations have also been shown to stabilize the proteins (Chen, 1980). After extensive dialysis against buffers containing EDTA, the D-xylose isomerase activity of the *Arthrobacter* enzyme showed absolute dependence on these ions (Fig. 5). Mg^{2+} ions are clearly preferred; studies with 2.5–10 mM-D-xylose and 50–150 μM-Mg^{2+} at 30 °C at pH 8 gave K_a for Mg^{2+} about 100 μM. Co^{2+} and Mn^{2+} ions were less effective, except that Mn^{2+} could substitute at very low metal ion concentrations. No activation was found with Ca^{2+}, Ba^{2+}, Zn^{2+} or Cu^{2+}.

Table 5 shows that Mg^{2+} and Mn^{2+} compete as activators for the active site of the enzyme, and other findings (not shown) show that Ca^{2+} is a competitive inhibitor with K_i about 2 μM. Similar metal-ion-binding studies have been conducted with the *Streptomyces violaceoruber* enzyme by Callens *et al.* (1988), which show two binding sites per protein monomer for the activating Co^{2+} or Mg^{2+} ions, the latter being the weaker. The binding of such ions in the tertiary structure of the *Arthrobacter* enzyme is discussed in detail by Collyer *et al.* (1990), and is an important factor in the mechanistic hypothesis that they propose.

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